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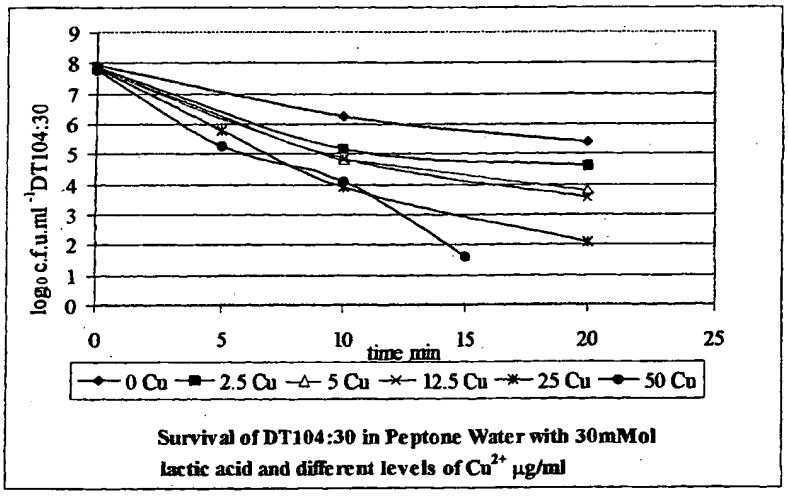
(54) Title: ANTIBACTERIAL COMPOSITION CONTAINING AN ORGANIC ACID AND COPPER SALT



Survival of DT104:30 in Peptone Water with 30mMol lactic acid and different levels of Cu^{2+} µg/ml

(57) Abstract: Provided are methods of controlling a target bacteria, which methods comprise the step of contacting them with an anti-bacterial composition comprising (i) a water soluble acid, and (ii) an added copper salt. This combination shows synergistic anti-bacterial effect. Preferred acids are organic acids selected from: acetic, lactic, propionic, and formic providing a pH of around 3.8 to 4.2 and whereby the copper is present at around 25 to 50 ppm. Methods may include applying such compositions to foodstuffs as surface decontaminants or other preservatives. Also provides are associated processes for producing anti-bacterial compositions, and the compositions (e.g. animal feeds or animal feed preservatives) themselves.

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ANTIBACTERIAL COMPOSITION CONTAINING AN ORGANIC ACID AND A COPPER SALT

Technical field

The present invention relates generally to methods and materials having anti-microbial activity, or for enhancing anti-microbial activity, particularly anti-bacterial activity.

Background art

A variety of anti-microbial compounds are known for use in controlling microorganisms, and particularly bacteria.

It is known that organic acids have antibacterial activity, with the effect usually attributed to pH depression arising from their ability to dissociate (as determined by their pKa value). Undissociated organic acids are lipophilic and can diffuse across the cell membrane. The lower the external pH, the more undissociated acid will cross the membrane. Various mechanisms have been proposed to explain how the acid then destroys bacterial cells. Traditionally, it has been considered that in the more alkaline interior of the cell the acids release protons that consequently lower internal pH. The elimination of the released protons by the membrane bound ATPases has traditionally been thought to result in the dissipation of the proton-motive forces which is essential for ATP synthesis and substrate uptake in the cell (Cherrington, Hinton, Mead and Chopra 1991). Alternatively, it has been proposed that the toxic effects of organic acids are caused by the accumulation of polar anions in the cells (Russell, Fletcher and Merka 1992). This accumulation depends on the pH gradient across the membrane. Bacteria that attempt to resist internal pH change are more sensitive to organic acids than those that allow pH to decline (Russell and Diez-Gonzalez 1998).

Disclosure of invention

The present inventors have demonstrated that the anti-microbial activity of organic acids can be significantly improved by the presence of copper salts. As shown in the Examples hereinafter, they have demonstrated that anti-bacterial action is unexpectedly both accelerated and increased by combinations of such acids and copper salt(s).

In its own right copper is not strongly antimicrobial. However, it was known that inclusion of copper (usually in the form of copper sulphate) in diets for pigs, at levels in excess of the normal nutritional requirement, have growth promoting effects (Bowland 1990; Hill, Cromwell, Crenshaw, Dove, Ewan, Knabe, Lewis, Libal, Mahan, Shurson, Southern and Veum 2000).

The underlying mechanism for the growth promoting properties of copper is and was unclear. A selective anti-microbial action on some elements of the gut microflora (Fuller, Newland, Briggs, Braude and Mitchell 1960) has been proposed as one mode of action. However, copper also appears to exert an effect through a variety of other mechanisms including stimulation of feed intake (Zhou, Kornegay, Vanlaar, Swinkels, Wong and Lindemann 1994); reduced turnover of intestinal mucosa (Radecki, Ku, Bennink, Yokoyama and Miller 1992); improvements to the villus architecture (Shurson, Ku, Waxler, Yokoyama and Miller 1990) resulting in increased digestion of fat (Dove 1995) and reduced maintenance requirement (Yen and Nienaber 1993). In addition high copper levels in the diet increased serum mitogenic activity (Zhou et al. 1994).

In the light of the results of the present inventors, it appears that the pH lowering effect of the acids of the prior art can be used not just directly for anti-microbial effect, as described above, but actually to **enhance** the anti-microbial activity of copper salts, giving an unexpectedly advantageous combination. In the examples below, combinations of acid and copper give enhanced activities where equivalent amounts of copper alone give no activity

at all. It is believed that the advantageous combination of copper ions and organic acids may be achieved using a wide range of acids against a wide range of organisms, particularly bacteria.

Recently, Alakomi et al. (2000) reported that lactic acid functions as a permeabilizer of the gram-negative bacterial outer membrane and may act as a potentiator of the effects of other anti-microbial substances naturally produced by lactic acid bacteria. No mention is made of copper as a potential additive for lactic acid, and in any case the results herein appear to be widely applicable to acids, including those which do not appear to have a permeabilizing effect.

Zimmerman (1966) J Bacteriol. 91(4) 1537-1541 suggested that copper plus ascorbic acid (at **neutral** pH) may exert an antibacterial affect against *Serratio marcescens* via a redox interaction with Fe-containing components of the cell. By contrast, the present invention is applied at lower pH's and employing non-autoxidisable acids.

In any case, those skilled in the art will appreciate that, irrespective of the precise mechanism for the unexpected synergy, the discovery has profound implications for the formulation and use of organic-acid based anti-microbials. In those instances where copper is already used, it may be possible to use reduced amounts with equivalent effect and hence environmental advantage.

GB 1574939 discusses the use of long branched-chain organic acids which are used as a means of impregnating wood and depositing metal salts as a fungicide. Unlike the present invention they do not suggest any synergistic antibacterial action of copper salts and water soluble organic acids.

WO 85/04668 A1 is also concerned with wood preservation against insects and funghi whereby copper is complexed with ammonia and the organic acid such that the resultant preservative is of neutral pH.

GB 1441232 discusses the use of nitric acids or related salts and

copper for reducing odours from pig and poultry manure. Unlike the present invention (which does not rely on nitric acid-base materials) there is no explicit disclosure of antibacterial action, synergistic or otherwise.

WPI abstract Acc. No 1996-514846 (JP 8268821) concerns chitosan and metal ions, wherein the lactic acid is used to dissolve the chitosan. However there is no disclosure of antibacterial action of the metal ions and the lactic acid.

WPI Abstract Acc. No 1989-167815 (HU 47812)discusses treatment of carcasses by immersion in lactic and nitric or phosphoric acid followed by a subsequent surface treatment of copper sulphate. The use of synergistic compositions comprising both organic acids and copper together are not disclosed.

WPI Abstract Acc. No 1988-143413 (JP 63084554) discusses deodorants containing *inter alia* iron which is alleged to have an antimicrobial effect. The present invention is not concerned with such iron-containing compositions.

WPI Abstract Acc. No 1987-217091 (JP 62142559) also discusses deodorizers, which are present in a fabric carrier. The present invention is not concerned with such deodorant fabric carriers.

Some aspects of the present invention will now be discussed in more detail.

Thus in a first aspect of the present invention there is provided a method of enhancing the anti-bacterial activity of an acid composition, which method comprises the step of introducing copper, preferably as a copper salt, into said composition. Use of copper to enhance the anti-microbial activity of an acidic composition is a further aspect.

It will be appreciated by those skilled in the art that it is the combination of acid and copper which has the unexpectedly effective

property, and where addition of one (say, copper) to the other (say, acid) is recited, the converse also applies.

The acid will be one which is soluble in aqueous solution. Preferably the acid is an organic acid which itself has an anti-microbial action. For example this may be selected from: acetic, dehydroacetic, citric, fumaric, lactic, malic, propionic, formic, succinic, sorbic and tartaric. Short chain carboxylic acids (C1-C10, more preferably C1-C6, most preferably C1-C3) are preferred.

Those skilled in the art will appreciate that where the term acid is used herein this does not require a single, pure acid, and that mixtures of acids (e.g. soluble, organic acids, especially short chain organic acids) may be used. Likewise the term 'acid' includes also the salts of weak acids, which will be present in both protonated and unprotonated form in accordance with their pK and the pH of use.

Most preferably the acid(s) will be selected from: acetic (ethanoic), lactic(2-hydroxypropanoic), propionic, and formic (methanoic) or a mixture of acid(s) and acid salt(s)

The enhancement may be by way of acceleration of the activity with respect to a particular target microorganism, or increase in effect for a lower concentration or amount of the organic acid. The enhancement is synergistic in the sense that the combined activity of the copper and organic acid greater than the sum of their respective activities. Preferably the enhancement is greater than or equal to 2, 3, 4, 5 or 10 fold.

The anti-microbial activity with respect to a particular target microorganism can be demonstrated using the methods described herein, or methods analogous thereto. For example a nutrient composition containing different concentrations of the organic acid and\or copper can be seeded with the target microorganism, and the numbers of viable microorganisms can be assessed at intervals and compared with an appropriate control. Such figures can be used to

generate a decimal reduction time (D_{value}) which is the time taken for the population to decrease by 90% and gives a measure of the effectiveness of an anti-microbial treatment.

The target microorganism may be any bacterium that is known or believed to be controlled by copper (and possibly organic acids, although use against organisms not controlled by organic acids may also be appropriate). Specific commercially relevant groups of microorganisms which may be controlled include, potential enteropathogens such as Salmonellas, Coliforms, Campylobacters, Listeria, Yersinia and Staphylococci, and spoilage organisms such as Pseudomonads, Bacillus spp, Clostridia. Preferred target bacteria are gram negative bacteria.

Specifically, the compositions of the present invention may be used to control one or more of the following groups of bacteria: Pseudomonads, especially *P.aeruginosa ; Propionobacter acnes, Pityosporium ovale, Pseudomonas cepacia;* Salmonella, especially *S. choleraesuis, S. enteriditis, S. typhimurium* and *S. goldcoast, S. anatum* and *S. derby,;* Streptococci, especially *S. mutans* and *S. suis; Escherichia coli* especially *E. coli,* O157 H7 and K88 185; Bacillus, especially *B. cereus* and *B. subtilis ;* Listeria, especially *L. monocytogenes* and *L. inocua;* and Proteus, especially *P. vulgaris.* Also of interest are Campylobacter, especially *C. jejuni* and Yersinia, especially *Y. enterocolitica.*

The anti-microbial compositions of the present invention will generally include further constituents appropriate to their use. Example compositions may be preserved foodstuffs (human or animal), surface decontaminants for use with foodstuffs, topically applied anti-microbials for use on humans or animals, sanitisation products, or anti-microbial additives for any of these. Organic acids are widely used as anti-microbial additives in both animal and human food industries (Doores 1993; Lambert and Strafford 1999). For example, organic acid washes and sprays are used to decontaminate carcasses in slaughterhouses and poultry processing plants (Dickson and Anderson 1992; VanNetten, Veld and Mossel 1994).

Example organic acid compositions to which the present invention may be applied are discussed in more detail below.

The pH of the final combination will preferably be close to the pKa value of the acid in question, preferably in the range pH 2-4.5, more preferably 3.8-4.2, more preferably about 4.

The methods or processes of the invention may include the step of adding the acid to a copper-salt containing composition of the type disclosed herein such as to achieve the appropriate final pH, and optionally using the resulting composition to control bacteria.

The preferred concentrations of components in the compositions described herein will depend, *inter alia*, on their use (e.g. the extent to which they will be diluted or mixed). Thus acid may be present prior to application at 0.01-99%.

Typically the organic acid will be present in the composition at a concentration (or to achieve a final concentration in use) of between 1-300 or 1-500 mMol. Compositions may include e.g. 25-500 mMol, for example greater than or equal to about 50, 100, 150, 200, 250, 300 mM and so on, depending on the organic matter content and buffering capacity of the target material. The organic acid may be present in the composition by in situ production e.g. by lactic acid bacteria. Indeed the present inventors have shown that 5mg Cu^{2+} per 100 ml medium, does not inhibit the growth of lactic acid bacteria.

The copper can be in the form of any soluble copper I or copper II salt, or organic forms of copper, in an appropriate concentration.

For surface sanitation the concentration will be that which gives optimum bacteriocidal effect. This will be determined by the microbiological and organic matter load. In embodiments where the composition is a feed of some sort, the copper will be present at over 'normal' requirement i.e. in excess of established nutritional requirements. In the Example 1 below 5mg Cu^{2+} per 100ml liquid feed

(equates to about 175mg Cu^{2+} per kg dry feed) showed significant effect. In other Examples levels as low as 25, 12.5, 5 and 2.5 μg ml^{-1} were also effective. Preferred compositions contain between 25-50 μgml^{-1} Cu^{2+} (or ppm Cu^{2+}, the units are used interchangeably herein).

Naturally, the precise optimum concentrations of acid and copper used may depend upon the nature of the composition in question, the precise identity organic acid used, the organic load, buffering capacity, regulatory issues etc. Nevertheless, in the light of the present disclosure, those skilled in the relevant art or arts will be able to apply the invention in appropriate manner.

Particularly preferred compositions of the present invention include:

Additives for use in solid animal (primarily pig and poultry) feeds. Preferably these will contain between 100-300 (e.g. 10-300) mMol organic acid and 1-250 (e.g. 25-75) μg g^{-1} Cu^{2+}. Higher levels of acids may be preferred, as appropriate to the buffering capacity of feed components.

Liquid feed ingredients and liquid feeds used as animal feed e.g. fermented liquid feeds fed to farm livestock and poultry Preferably these will contain between 10-300 (e.g. 50-300 mMol) organic acid (~0.1-3% or 0.5-3% depending on MW of acid) and between 12.5-75 (2.5-250) μg ml^{-1} Cu^{2+}, as appropriate to the buffering capacity and dry matter content of the feed.

Organic acid sprays used for the surface decontamination of meat (carcasses and portions). Preferably these will contain between 50-200 mMol organic acid and 2.5-50 μg ml^{-1} Cu^{2+}.

For preservatives for animal feed and human food based on lactic acid bacterial fermentations the lactic acid will already be present from the fermentation. Preferred levels of Cu^{2+} may be 1-20 μg ml^{-1} (human food) or 2.5-50 μg ml^{-1} (animal feed).

Immersion fluids and topical applications (sprays) for use as surface decontaminants for meat (e.g. beef, pig, sheep and poultry carcasses and meat portions (primal cuts, joints and further processed portions) vegetables (e.g. salad crops and root vegetables) and eggs.

Preferably these will contain between 5-200 (e.g. 50-200) mMol organic acid and 1-50 or 2.5-50 μg ml^{-1} Cu^{2+} (depending on Cu^{2+} residue, and also any regulatory limit of Cu^{2+} for vegetables, which may be in the region of 10-50 mg kg^{-1}).

The amount of the composition to be applied to a feedstuff to be preserved is suitably from 0.1-10%, preferably from 0.2-5% by weight of the total feedstuff. The compound animal feedstuff to which the preservative composition is applied may be in the pelleted or mash form. The preservative compositions of the present invention may be applied to diets for pigs, cattle, sheep, poultry, , ostrich, game birds, fish,aquatic species fur-bearers, camelids, equines, laboratory, zoo and companion animals. Poultry, pigs and laboratory animals are particularly susceptible to salmonella infection from the feed and the compositions of the present invention are especially suited to mitigating the effects of salmonella in such feeds. Typically, the animal feed will have the following composition: (w/w % - Cereals and by-products (50-80); Vegetable proteins (0-30); Animal proteins (0-15); Miscellaneous (0-25)). In addition the invention has particular application in the preservation of liquid food and feed components and in liquid diets for animals.

Other embodiments include:

Anti-microbial surface application to grains, pulses and pelleted feeds. Preferably these will contain 50 - 500mM acid and up to 100 $\mu g\ ml^{-1}$ copper.

Water treatment; applications for the reduction of microbial load in water supplies used for animal consumption and for cleaning purposes. Preferably these will contain 5 - 200mM acid and up to 100 $\mu g\ ml^{-1}$ copper in the final preparation.

Effluent treatment; Acids are added to human and animal effluent (sewage) to reduce the microbial load and to reduce odour.

Anti-microbial washes/baths for topical application (e.g. foot-baths for the prevention / treatment of hoof problems in farm livestock and immersion products for the treatment of athletes foot in humans). Preferably these will contain between 100-300 mMol organic acid and up to around 25 mg ml^{-1} Cu^{2+}.

Hygiene products for use on mucous membranes (e.g. mouthwashes and douches). Preferably these will contain between 25-200 mMol organic acid and 2.5-25 $\mu g\ ml^{-1}$ Cu^{2+}.

Sanitisation products for use on surfaces (e.g. household disinfection, cleaning of food preparation surfaces, cleaning pipelines in food manufacture). Preferably these will contain between 50-300 mMol organic acid and up to 100 (say, around 12.5-75) $\mu g\ ml^{-1}$ Cu^{2+}.

Foliar sprays. Preferably these will contain between 25-200 mMol organic acid and up to 2 mg ml^{-1} Cu^{2+} (which is a current recommended level per ha).

In a further aspect of the present invention there is provided a method of producing an organic acid composition, particularly any of those discussed above, having enhanced anti-microbial activity,

which method comprises the step of introducing copper into said composition, or into a fermentation process for producing said composition.

As stated above, in the preparation of the advantageous composition, the order in which the components (organic acid, copper, other ingredients) are introduced is not important, although the invention may have particular utility in supplementing any copper present e.g. in commercial compositions in which organic acids are already present as anti-microbials.

Compositions *per se*, as described above, form a further aspect of the present invention. Also embraced are such compositions for use in medical or veterinary treatments, and the use of the compositions in the manufacture of medical or veterinary compositions for treatments of the human or animal body.

The invention particularly provides use of a composition as described above to control microorganisms on or in a subject or substrate to which the composition is administered or applied. By "control" is meant that the composition prevents or inhibits the growth of one or more microorganisms such as bacteria and fungi. This may include the killing of or the prevention of growth of microorganisms and/or a reduction or prevention of the production of microbial metabolites which may have an adverse effect on the composition, structure, appearance, taste, flavour, smell, or safety of the material in which they are disposed.

Control may be effected by administration of the composition e.g. orally, topically, etc.

The invention will now be further described with reference to the following non-limiting Figures and Examples. Other embodiments of the invention will occur to those skilled in the art in the light of these.

FIGURES

Figure 1: Survival of *S. typhimurium* DT104:30 in liquid pig feed treated with lactic acid in the presence or absence of copper sulphate.

Figure 2: Survival of *S. typhimurium* DT104:30 in liquid pig feed treated with organic acids and/or zinc or copper salts, treatments as per legend.

Figures 3, 4 and 5: survival curves of *S. typhimurium* DT104:30 in acidified peptone water and skimmed milk acidified with lactic acid and HCI are presented in respectively.

EXAMPLES

Example 1

Fermenting liquid feed for pigs with lactic acid bacteria results in a feed that contains between 15^ and 250 mMol lactic acid and has a pH of 3.8 - 4.0. This imparts anti-microbial properties to FLF, which enables it to resist contamination by other microorganisms such as enteropathogens.

A first study was conducted to assess the affect of lactic acid inclusion level in the presence or absence of copper sulphate on the decimal reduction time (D_{value}) of *S. typhimurium* DT104:30 in liquid pig feed. The D_{value} is the time taken for the population to decrease by 90% and gives a measure of the effectiveness of an anti-microbial treatment.

Sterile liquid pig feed was prepared by adding feed that had been irradiated (γ irradiation with Cobalt 60), to eliminate the natural microflora, to sterile distilled water in a ratio of 1 part feed to 2.5 parts water. This was dispensed into 100ml aliquots. Three replicate samples of each of the following treatments of the resultant liquid feed were prepared.

Liquid feed plus:

1. 50 mMol Lactic acid
2. 50 mMol Lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
3. 100 mMol Lactic acid
4. 100 mMol Lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
5. 150 mMol Lactic acid
6. 150 mMol Lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
7. 200 mMol Lactic acid
8. 200 mMol Lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
9. 300 mMol Lactic acid
10. 300 mMol Lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)

The level of copper sulphate added to the liquid feeds equated to that commonly added to weaner pig diets, i.e. to give 175mg Cu^{2+} per kg dry feed.

All samples were equilibrated to 30°C in a water bath, after which they were seeded with an inoculum of approximately 10^6 c.f.u. ml-1 *Salmonella typhimurium* DT104:30. The survival of *S. typhimurium* DT104:30 in the liquid feed samples was determined as follows.

The number of viable *S.typhimurium* DT104:30 remaining in the feed were enumerated at hourly intervals for five hours following inoculation. Samples (1 ml) of each feed were serially diluted in buffered peptone water and appropriate dilutions were plated onto blood agar agar plates using a standard spread plate method. The agar plates were incubated at 37°C for 24 h after which viable *S. typhimurium* DT104:30 were enumerated. This allowed the decimal reduction of *S. typhimurium* DT104:30 in each feed treatment to be calculated.

Results

The D_{values} obtained for the liquid feed treatments are presented in Table 1. The survival curves for *S. typhimurium* DT104:30 in each liquid feed treatment are presented in Figure 1. The D_{value} of *S. typhimurium* DT104:30 decreased with increasing levels of lactic acid. The addition of copper sulphate further decreased the D_{value} at all levels of lactic acid inclusion. However, the effect of copper sulphate was most apparent at lactic acid inclusion levels of 150 mMol where there was a 10 fold decrease in the D_{value} compared with 2 - 4 fold decreases at other lactic acid levels.

Table 1 Decimal reduction time of *S. typhimurium* DT104:30 in liquid pig feed treated with lactic acid and copper sulphate (5mg Cu^{2+} per 100ml feed).

Liquid Feed Treatment	D_{value} of *S. typhimurium* DT104:30 (min)	SD
50 mMol Lactic acid	389[a]	31.4
50 mMol Lactic acid + copper sulphate	121[b]	8.9
100 mMol Lactic acid	383[a]	92.5
100 mMol Lactic acid + copper sulphate	86	20.6
150 mMol Lactic acid	334[a]	56.0
150 mMol Lactic acid + copper sulphate	33[c]	1.28
200 mMol Lactic acid	131[b]	6.38
200 mMol Lactic acid + copper sulphate	32[c]	3.19
300 mMol Lactic acid	19	4.1
300 mMol Lactic acid + copper sulphate	7.5	0.2

[a,b,c] means with the same superscript are not significantly different (P>0.05)

The results are shown in Figure 1. As can be seen, it appears that there is a synergistic relationship between copper sulphate and lactic acid whereby the addition of copper sulphate enhanced the microbial activity of lactic acid against *S. typhimurium* DT104:30.

Example 2

A second study was conducted to demonstrate that the synergistic effect observed in the first study was true for other copper salts, metal sulphates or organic acids. Copper chloride was used as an alternative copper salt, zinc sulphate as an alternative sulphate

source and acetic acid as an alternative organic acid. In addition the effect of copper sulphate alone and liquid feed with no additional treatment on the survival of *S. typhimurium* DT104:30 were included in this study. Feed was prepared as in the previous study, and three replicate samples of each of the following treatments of the resultant liquid feed were prepared.

Liquid feed plus:-

1. no acid or metal salts (control)
2. copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
3. 150 mMol lactic acid
4. 150 mMol lactic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)
5. 150 mMol lactic acid + copper chloride (to give 5mg Cu^{2+} per 100ml liquid feed)
6. 150 mMol lactic acid + zinc sulphate (to give 5mg Zn^{2+} per 100ml liquid feed)
7. 150 mMol acetic acid
8. 150 mMol acetic acid + copper sulphate (to give 5mg Cu^{2+} per 100ml liquid feed)

All samples were equilibriated to 30°C in a water bath, after which they were seeded with an inoculum of approximately 106 c.f.u. ml *Salmonella typhimurium* DT104:30. The survival of *S. typhimurium* DT104:30 in the liquid feed samples was determined as follows.

The number of viable *S.typhimurium* DT104:30 remaining in the feed were enumerated at hourly intervals for three hours following inoculation by the method outline in study 1.

Results

The survival curves for *S. typhimurium* DT104:30 in each feed treatment are presented in Figure 2 The decimal reduction times, calculated as the reciprocal of the slope of the survival curve are presented in Table 2. In liquid feed with no addition of acid or metal salts or with copper sulphate only there was no reduction in

the population of *S. typhimurium* DT104:30. The addition of either 150mMol lactic or acetic acid resulted in decimal reduction times of 350 and 431 min respectively. The addition of zinc sulphate to liquid feed treated with lactic acid did not result in any further increase in the decimal reduction time of *S. typhimurium* DT104:30. However, the addition of copper salts either as copper sulphate or copper chloride to liquid feed treated with lactic acid significantly reduced the decimal reduction time of *S. typhimurium* DT104:30 to 39 min and 33 min respectively. Similarly, the addition of copper sulphate to feed treated with acetic acid reduced the decimal reduction time to 37 min. These results show that the addition of copper salts to liquid pig feed acidified with lactic or acetic acid reduces the decimal reduction time of *S. typhimurium* DT104:30 by approximately 10 fold.

Table 2. Decimal reduction time of *S. typhimurium* DT104:30 in liquid pig feed treated with lactic or acetic acid and metal salts.

Feed treatment	**Decimal reduction time of *S. typhimurium* DT104:30 min**	S.D
150 mMol lactic acid	350b	104
150 mMol lactic acid + copper sulphate	39a	3.5
150 mMol lactic acid + copper chloride	33a	2.0
150 mMol lactic acid + zinc sulphate	347b	44.0
150 mMol acetic acid	431	61.0
150 mMol acetic acid + copper sulphate	37a	1.4

$2^{a,b}$ means are not significantly different P> 0.05

The results are shown in Figure 2.

These Examples show that the ability of a microorganism (here *S. typhimurium* DT104:30) to survive in a composition (here liquid pig feed) that has been acidified by organic acids is greatly reduced in the presence of copper salts. Indeed the anti-microbial effect of organic acids was enhanced 10 fold by the addition of copper salts in instances (see Figure 1) where the copper per se was not significantly anti-microbial. These results are particularly significant given that the enteric pathogen *S. typhimurium* DT104:30 is highly able to withstand environmental stressors such as acid conditions.

Example 3

This Example shows the effect of different levels of copper sulphate on the survival of *S. typhimurium* DT104:30 in peptone water and skimmed milk acidified with lactic acid.

Studies were conducted using defined media rather than liquid pig feed in order to control any variability arising from the range of raw materials present batch to batch in pig feed.

Peptone water, a minimal media that will support the survival of bacteria, but that has no particulate organic material was used. Skimmed milk was used as a replicable source of an organic material. The pH of skimmed milk acidified with 100 mMol lactic acid was 4.12. In order to ascertain whether the effect of Cu^{2+} addition was due to decreased pH a further study was conducted using skimmed milk acidified to pH 4.12 with hydrochloric acid.

The survival of *Salm. typhimurium* DT104:30 was determined in the following media:-

- peptone water (Oxoid Ltd) acidified with 30 mMol lactic acid with additions of copper sulphate to give 50, 25, 12.5, 5 2.5 and 0 μg ml^{-1} Cu^{2+}

- skimmed milk (Oxoid Ltd) acidified with 100 mMol lactic acid, with additions of copper sulphate to give 50, 25, 12.5, 5 2.5 and 0 μg ml^{-1} Cu^{2+}

- skimmed milk (Oxoid Ltd) acidified with hydrochloric acid to pH 4.12, with additions of copper sulphate to give 50, 25, 12.5, 5 2.5 and 0 μg ml^{-1} Cu^{2+}

Method

As lactic acid is itself antimicrobial an initial investigation was conducted to determine the level of inclusion of lactic acid that reduced a population of *Salm. typhimurium* DT104:30 by four log_{10} units in one hour. The level required was 30 mMol lactic acid and 100 mMol lactic acid in peptone water and skimmed milk respectively. Similarly, an initial investigation demonstrated that copper sulphate alone had no effect on the D_{value} of *Salm. typhimurium* DT103:30 in 90 min.

Study 1

Peptone water (50 ml) was acidified with 30 mMol lactic acid. Copper sulphate was added to give final concentrations of Cu^{2+} of 50, 25, 12.5, 5, 2,5 and 0 μg ml^{-1}. Three replicate samples of each were inoculated with approximately 10^8 c.f.u. ml^{-1} of *Salm. typhimurium* DT104:30 and incubated at 30°C. Samples were taken at appropriate intervals after inoculation, diluted in buffered peptone water and plated onto brain heart infusion agar using a spiral plater (Don Whitley Ltd). The plates were incubated at 37°C for 24 h after which the number of viable *Salm. typhimurium* DT104:30 were counted using a laser plate counter.

Study 2

Skimmed milk was acidified with 100 mMol lactic acid and the study was conducted as above.

Study 3

The pH of skimmed milk was adjusted to pH 4.12 (± 0.02) with hydrochloric acid, and the study was conducted according to the method described in Study 1.

Survival curves were constructed from the data and the D_{value} calculated as the reciprocal of the slope of the survival curve. Individual D_{values} were analysed by analysis on variance (one-way) for each media.

Results

The survival curves of *Salm. typhimurium* DT104:30 in acidified peptone water and skimmed milk acidified with lactic acid and HCI are presented in Figures 3, 4 and 5 respectively. The D_{value} are presented in Table 3.

Table 3 D_{value} of *Salm. typhimurium* DT104:30 in Peptone Water (PW) acidified with 30 mMol Lactic acid, Skimmed Milk (SM) acidified with 100 mMol Lactic acid or Skimmed Milk acidified with hydrochloric acid to pH 4.12. (Standard deviations are given in brackets)

	PW + 30 mMol lactic	SM + 100 mMol lactic	SM + HCl(pH 4.12)
0µg ml^{-1} Cu^{2+}	7.8 (1.2)	106.5 (13.7)	385.5(67.8)
2.5µg ml^{-1} Cu^{2+}	6.1^{b} (0.2)	20.2^{a} (0.5)	65.6^{a} (4.6)
5µg ml^{-1} Cu^{2+}	4.9^{ab} (0.5)	10.7^{ab} (1.6)	34.4^{a} (4.2)
12.5µg ml^{-1} Cu^{2+}	4.5^{a} (0.7)	7.9^{ab} (0.5)	12.9^{a} (2.2)
25 µg ml^{-1} Cu^{2+}	4.3^{a} (0.1)	3.6^{b} (0.4)	5.5^{a} (0.7)
50 µg ml^{-1} Cu^{2+}	2.5 (0.1)	2.4^{b} (0.8)	4.3^{a} (0.3)
s.e.d.	0.48	4.6	22.7

Conclusions

The addition of Cu^{2+} to media acidified with either lactic acid or hydrochloric acid increased the death rate of *S. typhimurium* DT 104:30 in the medium. The fact that an effect was observed with a combination of Cu^{2+} and hydrochloric acid suggests that the enhancement of antimicrobial activity may, at least in part, be due to the presence of Cu^{2+} at low pH.

The results demonstrate that, although much higher levels of lactic acid are needed to kill *S. typhimurium* DT104:30 in the presence of organic matter in SM compared with PW, the addition of Cu^{2+} enhances the antimicrobial effect of the acid.

In skim milk acidified with lactic acid or hydrochloric acid the present of low levels of Cu^{2+} (2.5 μg ml^{-1}) increase the death rate of *S. typhimurium* DT104:30 by a factor of 45 and 90 respectively.

Example 4

This describes the effect of the presence of copper on the survival of various types of bacteria in acidified skimmed milk.

Different strains of bacteria as described below were screened for their sensitivity to 50ppm copper, 100 mM lactic acid and a combination of the two in skimmed milk by measuring the percentage of viable organisms surviving after exposure to each treatment for one hour at 30°C.

Skimmed milk was inoculated with of each of the above organisms. The number of viable organisms were enumerated immediately prior to treatment of the skimmed milk samples with the following:-

1 50 ppm copper from copper sulphate,

2 acidification with lactic acid to give a final concentration of 100mM,

3 acidification to 100 mM with lactic acid plus 50 ppm copper.

Each treatment was conducted in triplicate for each organism. Samples were incubated for one hour at 30°C after which the numbers of viable organisms remaining were enumerated.

The survival of *S. typhimurium* DT104:30 and E.coli O157 H7 in skimmed milk treated with acetic, propionic and formic acids in the presence or absence of 50 ppm copper was assessed. Skimmed milk was inoculated as described previously and treated with each acid alone to give a final concentration of 100mM, or with each acid plus 50 ppm copper from copper sulphate. Viable organisms were enumerated immediately prior to treatment and an hour after treatment and incubation at 30°C for one hour.

Results

The results of the numbers of organisms surviving each treatment (expressed as $\log_{10}$) are presented in Table 4. The presence of 50 ppm copper alone had no significant affect on the survival of any of the organisms tested. In skimmed milk containing 100 mM lactic acid alone, there was no significant reduction in numbers of *Staph. aureus, Y. enterocolitica* or *E. coli* K88 185. However, lactic acid alone had some degree of antimicrobial activity towards the other organisms. The addition of 50 ppm copper to skimmed milk containing 100 mM lactic acid had a significant affect of the survival of all organisms except *Staph. aureus* over and above the effect of lactic acid alone. However, whilst the addition of 50 ppm copper resulted in a 10^3 - 10^5 fold decrease in the number of Gram negative organisms (*E. coli, Salmonella* and *Yersinia*) compared with lactic acid alone (Table 5) there was only a 5 fold decrease in numbers of *L. inocua* (Gram positive). Although still showing an effect, this suggests that Gram positive organisms (*Staph* and *Listeria*) may be less sensitive to copper in acidic substrates than Gram negative organisms.

The presence of 50 ppm copper increased the antimicrobial effect of acetic, propionic and formic acids. Formic acid was the most

effective in reducing numbers of *E. coli* O157 H7 and *S. typhimurium* DT104:30 after exposure for one hour at 30°C, reducing the percentage of organisms surviving by a factor of 10^4 to 10^5. In comparison the addition of 50 ppm copper to acetic and propionic acids reduced the % organisms surviving by 10 - 100 fold (Tables 6 - 8). This difference may be due to pH differences in the skimmed milk due to different acidifying properties of the organic acids. The pH of skimmed milk containing 100 mM of acetic, propionic and formic acids was 4.39, 4.44 and 3.89 respectively. In comparison the pH of skimmed milk containing 100mM lactic acid was 4.12. Therefore the ability of copper/organic acids to act as an antimicrobial is can be pH dependant.

Table 4 Log_{10} numbers of organisms surviving for 1 hour at 30°C in skimmed milk containing 100mM lactic acid, 50 ppm copper from copper sulphate or 100 mM lactic acid plus 50 ppm copper. S. *typhimurium* DT104:30 included for comparison from previous data (n=3)

	100 mM lactic acid		**50 ppm copper**		**100mm lactic acid + 50 ppm copper**	
Time (h)	0	1	0	1	0	1
S. aureus	5.09[a]	6.23[a]	5.46[a]	6.74[a]	5.88[a]	6.03[a]
L. inocua	7.07[a]	7.04[a]	7.06[a]	7.12[a]	7.10	6.34
Y. enterocolitica	6.77[a]	6.58[a]	6.80[a]	6.91[a]	6.82	0.98
E. coli O157H7	7.01	5.67	6.87[a]	7.24[a]	7.05	1.80
E. coli K88 185	7.07[a]	6.94[a]	7.06[a]	6.99[a]	7.10	1.47
S. derby	6.76	5.77	6.71[a]	7.14[a]	6.94	2.24
S. goldcoast	7.36	6.51	7.33[a]	7.15[a]	7.30	3.57
S. anatum	7.28	6.37	7.32[a]	7.29[a]	7.31	3.16
S. typhimurium DT104:30	7.26	6.36	7.39[a]	7.36[a]	7.29	1.90

[a] means with the same superscript within the same treatment are not significantly different $P > 0.05$

Table 5 Percentage of organisms surviving for one hour in skimmed milk treated with 100mM lactic acid, 50 ppm copper or 50ppm Copper + 100 mM lactic acid

Organism	100mM lactic acid	50 ppm Copper	100mM lactic acid + 50 ppm copper
Staph. aureus	100	100	100
L. inocua	88	100	16.5
Y. enterocolitica	65	100	0.0001
E. coli O157 H7	5	100	0.0006
E. coli K88 185	74	90	0.0002
S. derby	10	100	0.002
S. goldcoast	14	80	0.02
S. anatum	12	92	0.007
S. *typhimurium*	6	94	0.004

Table 6 Log_{10} numbers of organisms in skimmed milk containing 100 mM acetic acid in the presence or absence of 50 ppm copper from copper sulphate

Organism	no copper			+ 50 ppm copper		
	0 h	1 h	% survival	0 h	1 h	% survival
E. coli O157 H7	6.91	6.67	57.7	6.97	5.24	1.8
S. *typhimurium* DT104:30	7.34	6.90	63.7	7.34	5.21	0.7

Table 7 Log_{10} numbers of organisms in skimmed milk containing 100 mM propionic acid in the presence or absence of 50 ppm copper from copper sulphate

Organism	no copper			+ 50 ppm copper		
	0 h	1 h	% survival	0 h	1 h	% survival
E. coli O157 H7	7.06	6.91	71.4	7.07	5.59	3.4
S. *typhimurium* DT104:30	7.20	7.15	77.9	7.42	5.53	1.3

Table 8 Log_{10} numbers of organisms in skimmed milk containing 100 mM formic acid in the presence or absence of 50 ppm copper from copper sulphate

Organism	no copper			+ 50 ppm copper		
	0 h	1 h	% survival	0 h	1 h	% survival
E. coli O157 H7	6.96	6.44	29.4	7.14	2.70	0.003
S. *typhimurium* DT104:30	7.26	5.97	5.13	7.31	1.15	<0.001

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Claims

1 A method of controlling a target microorganism which is a bacterium, which method comprises the step of contacting the microorganism with an anti-bacterial composition comprising (i) a water soluble acid, and (ii) an added copper salt.

2 A method as claimed in claim 1 wherein the acid is an organic acid.

3 A method as claimed in claim 2 wherein the organic acid is selected from: acetic, dehydroacetic, citric, fumaric, lactic, malic, propionic, formic, succinic, sorbic and tartaric.

4 A method as claimed in any one of the preceding claims wherein the target microorganism is selected from Pseudomonads; Salmonella; Streptococci; Bacillus; Listeria; Proteus; Campylobacter; Yersinia.

5 A method as claimed in claim 4 wherein the target microorganism is one shown in Table 4.

6 A method as claimed in any one of the preceding claims wherein the organic acid is present in the composition at between 25-500 mMol.

7 A method as claimed in claim 6 wherein the organic acid is present in the composition at between 150-250 mMol.

8 A method as claimed in any one of claims 2 to 7 wherein the pH of the composition is approximately equivalent to the pKa value of the acid

9 A method as claimed in any one of the preceding claims wherein the pH of the composition is in the range 2-4.5.

10 A method as claimed in claim 9 wherein the pH of the composition is in the range 3.8 to 4.2.

11 A method as claimed in any one of the preceding claims wherein the copper is present in the composition at greater than about 1 to 75 μg ml^{-1}.

12 A method as claimed in claim 11 wherein the copper is present in the composition at between 25 and 50 μg ml^{-1}.

13 A method as claimed in claim wherein the microorganism is controlled on or in a subject or substrate in or to which the composition is present, administered or applied.

14 A method as claimed in claim 13 wherein the subject is a human or animal

15 A method as claimed in claim 14 wherein the administration of the composition is oral or topical.

16 A method as claimed in claim 13 wherein the substrate is foodstuff.

17 A method as claimed in any one of claims 13 to 16 wherein the composition is selected from: a surface decontaminant for use with a foodstuff; an additive for use with solid animal feed; topically applied anti-microbials for use on a human or animal.

18 A process for producing an anti-bacterial organic acid composition having enhanced anti-bacterial activity, which method comprises the step of introducing a copper salt into said composition, wherein the pH of the composition is in the range 3.8 to 4.2, and wherein the copper is present in the composition at between 25 and 50 μg ml^{-1}.

19 A process as claimed in claim 18 wherein the organic acid is produced *in situ* in the composition.

20 A composition as described in any one of claims 1 to 19 having enhanced anti-bacterial activity or resistance, which composition comprises (i) an organic acid, and (ii) an added copper salt.

21 A composition as claimed in claim 20 which is an animal feed or animal feed preservative.

22 A composition as claimed in claim 21 which is a fermented liquid animal feed.

23 A composition as claimed in claim 21 or claim 22 wherein the amount of the preservative used with or present in the feed to be preserved is from 0.1-10%, preferably from 0.2-5% by weight of the total feed.

24 A composition as claimed in any one of claims 21 to 23 wherein the animal is selected from: pigs, cattle, sheep, poultry, ostriches, game birds, equines, aquatic species (shellfish etc) fish, camelids, fur-bearers, laboratory, zoo and companion animals.

25 A composition as claimed in any one of claims 21 to 24 wherein the feed has the following composition (w/w %) - Cereals and by-products (50-80); Vegetable proteins (0-30); Animal proteins (0-15); Miscellaneous (0-25)).

26 A composition as claimed in claim 20 for use in a medical or veterinary treatment for the control of a bacterium.

27 Use of a composition of claim 20 in the manufacture of medical or veterinary compositions for treatments of the human or animal body for the control of a bacterium.

Figure 1

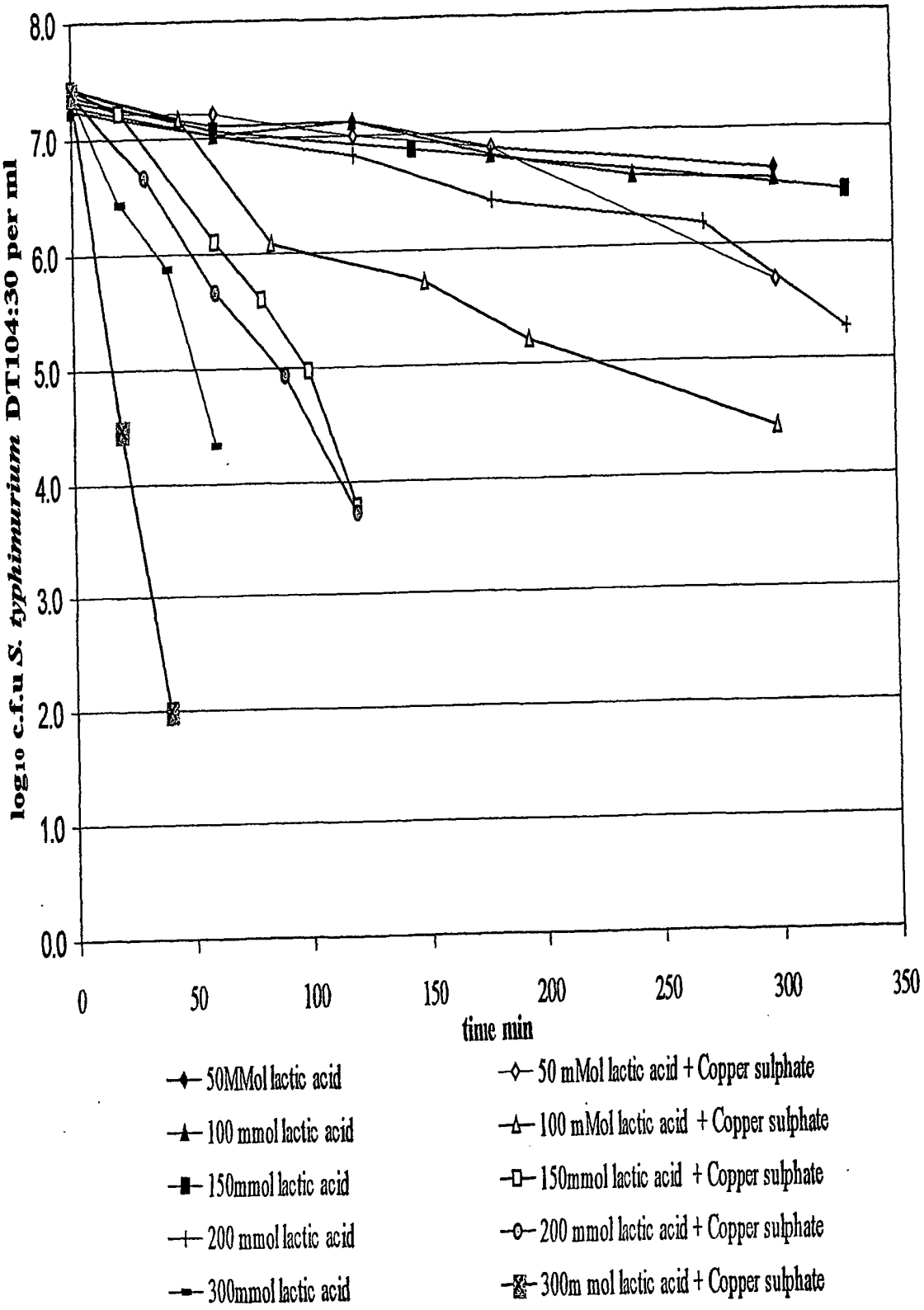

Figure 1 Survival of *S. typhimurium* DT104:30 in liquid pig feed treated with lactic acid in the presence or absence of copper sulphate.

Figure 2

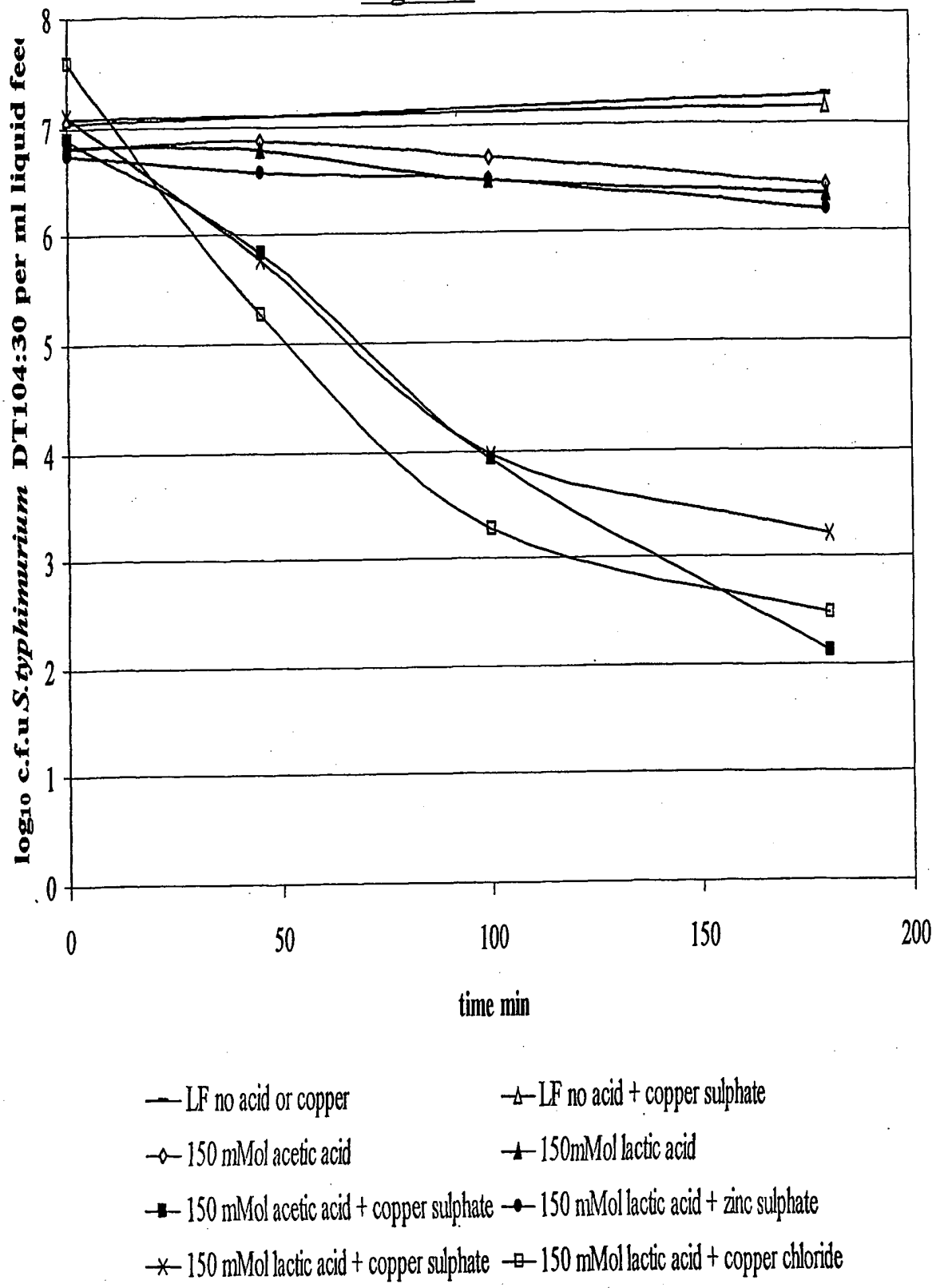

Figure 2 Survival of *S. typhimurium* DT104:30 in liquid pig feed treated with organic acids and/or zinc or copper salts, treatments as per legend.

Figure 3

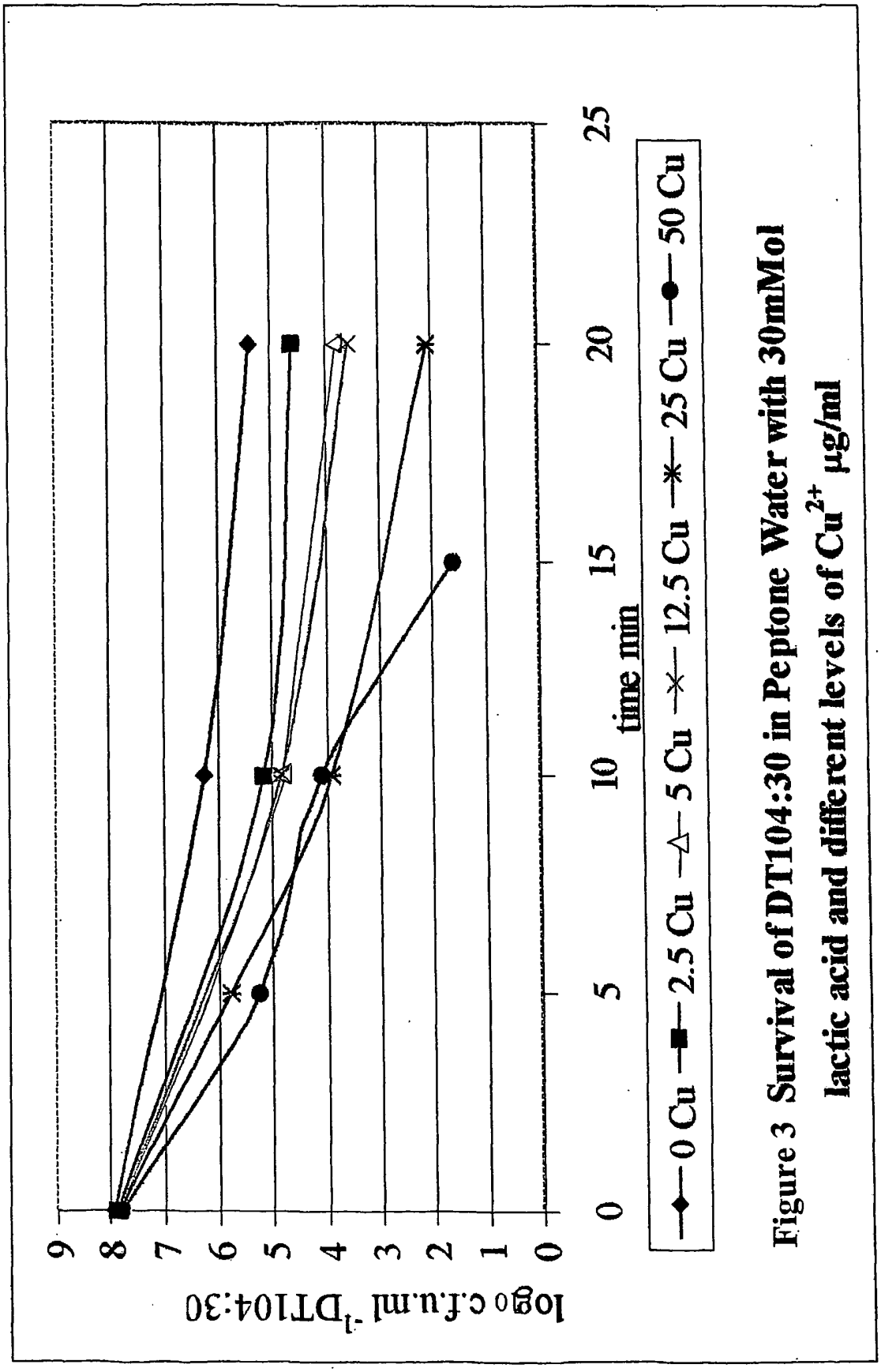

Figure 3 Survival of DT104:30 in Peptone Water with 30mMol lactic acid and different levels of Cu^{2+} µg/ml

Figure 4

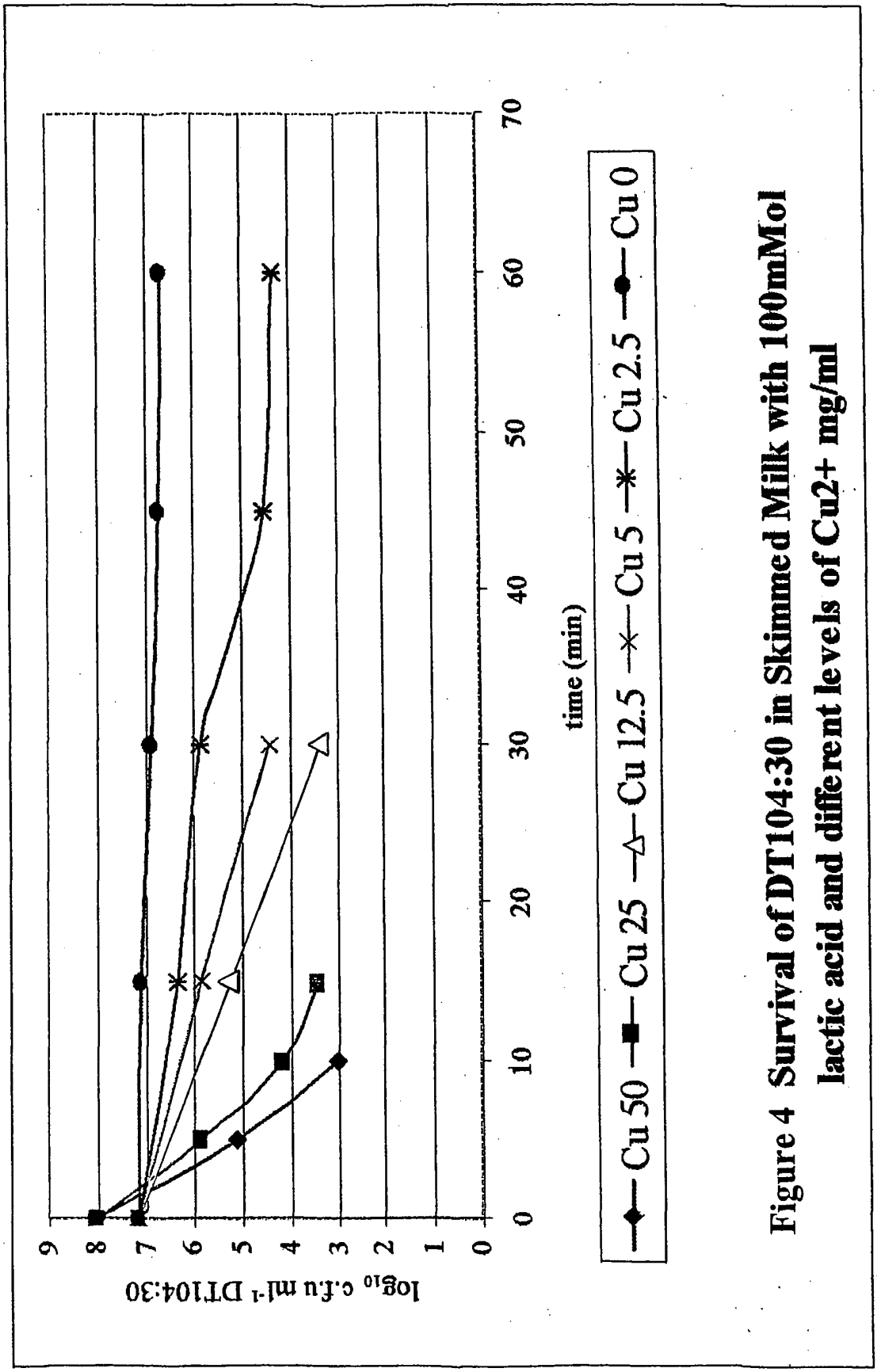

Figure 4 Survival of DT104:30 in Skimmed Milk with 100mMol lactic acid and different levels of Cu2+ mg/ml

Figure 5

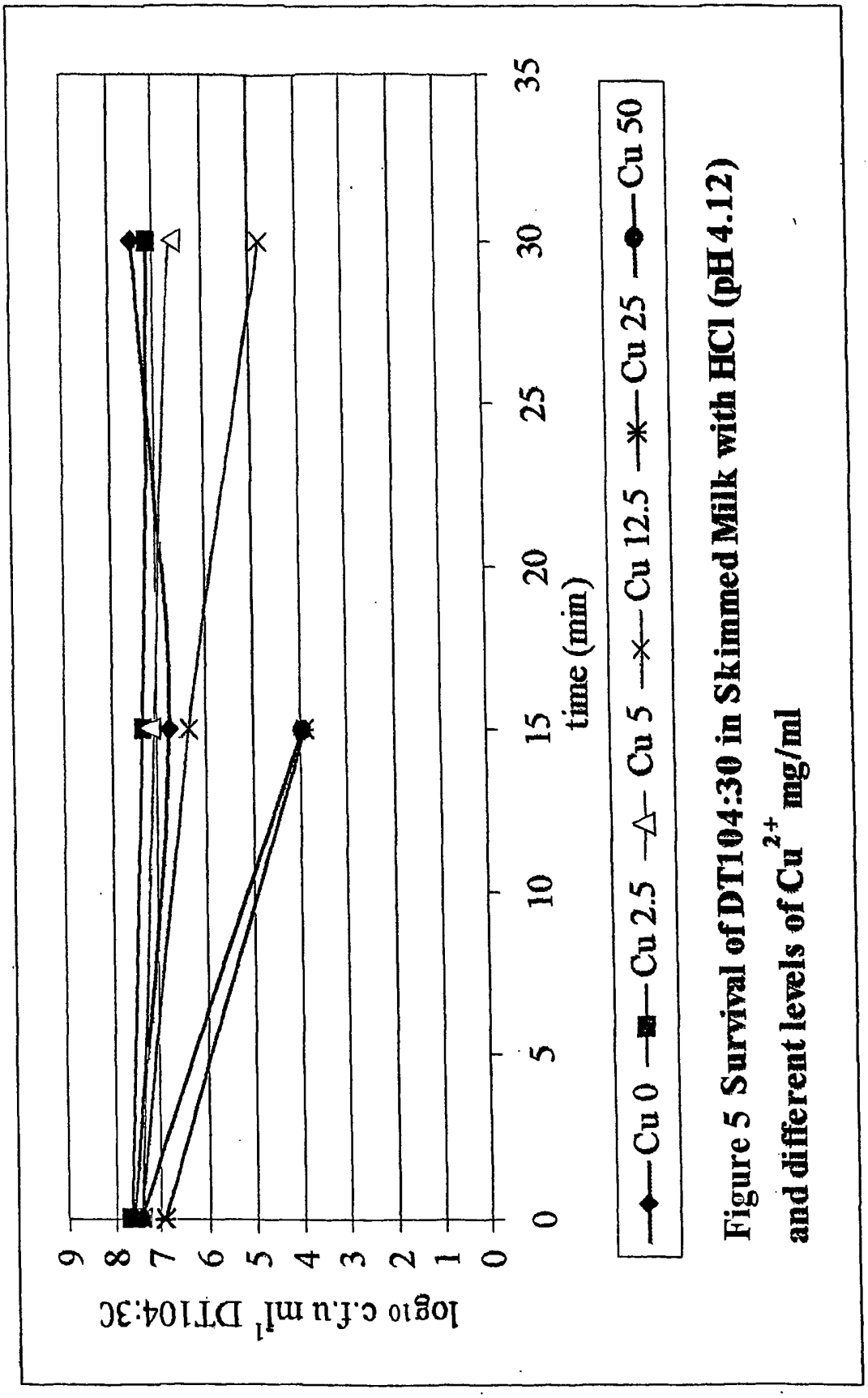

Figure 5 Survival of DT104:30 in Skimmed Milk with HCl (pH 4.12) and different levels of Cu^{2+} mg/ml

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02492

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A01N59/20 A01N37/02 A01N37/36 A01N37/06 A01N43/16
A61K31/19 A61K33/34 A23L3/3508 A23K1/175 //A01N59:20,
37:02,37:06,37:36,43:16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A01N A61K A23L A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Derwent Publications Ltd., London, GB; AN 1987-217091 XP002209284 "Antibacterial, deodorising material-consists of metal salt e.g. copper sulphate and organic acid with carrier e.g. nonwoven fabric" & JP 62 142559 A (SHOKO KAGAKU KENKYU), 25 June 1987 (1987-06-25) cited in the application abstract ---	1-3,13, 20
X	US 3 329 607 A (ANDRE CIER ET AL) 4 July 1967 (1967-07-04) column 2, line 60 - line 68 claims 1-5; examples 1-4,6 --- -/--	1-5,11, 13,16,20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 August 2002	04/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Nopper-Jaunky, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 997 911 A (MOURNING JACKIE BRINTON ET AL) 7 December 1999 (1999-12-07) column 1, line 61 - line 62 column 7, line 24 - line 64; claims 1,12-15; example 1	1,2, 13-16, 20,26,27
X	WO 86 07081 A (OSMOSE WOOD PRESERVING CO: OF AMERICA, INC.) 4 December 1986 (1986-12-04) cited in the application examples 1,3,6,9,11,12	20
X	US 4 797 274 A (MIKI YOSHIAKI ET AL) 10 January 1989 (1989-01-10) examples 2-1; table 1	20
X	WO 00 62609 A (AGRICARE LTD.) 26 October 2000 (2000-10-26) page 9; example B; table 5B	1-5,13, 20
X	US 4 490 389 A (NELSON ERIC L ET AL) 25 December 1984 (1984-12-25) claim 1; example V	20
X	US 4 743 454 A (TOMES NANCY J) 10 May 1988 (1988-05-10) claims 1,9,10; table III column 4, line 15	20
X	EDMONDS M.S., IZQUIERDO O.A., BAKER D.H.: "Feed Additive with Newly Weaned Pigs: Efficacy of Supplemental Copper, Antibiotics and Organic Acids" JOURNAL OF ANIMAL SCIENCE, vol. 60, no. 2, 1985, pages 462-469, XP002209307 page 467; table 7	20,21, 23-25
A	DATABASE WPI Derwent Publications Ltd., London, GB; AN 1999-613739 XP002209285 "Antimicrobial additive for fodder-contains ferrous sulfate, sodium propionate and/o propionic acid calcium and copper sulfate" & JP 11 266796 A (ZENBI SHOJI KK; FUJI KAGAKU KK; MORITA SHIGEYUKI), 5 October 1999 (1999-10-05) abstract	1-27

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	VANDERWAL P.: "Salmonella Control of Feedstuffs by Pelleting or Acid Treatment" WORLD'S POULTRY SCIENCE JOURNAL. , vol. 35, 1979, pages 70-78, XP002209392 *the whole document*	1-27
A	EP 0 260 390 A (DEGUSSA) 23 March 1988 (1988-03-23) *The whole document*	1-27
A	US 3 937 814 A (DARACK JOHN R ET AL) 10 February 1976 (1976-02-10) column 1, line 9 - line 17; claims 1-5	1-27
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